

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

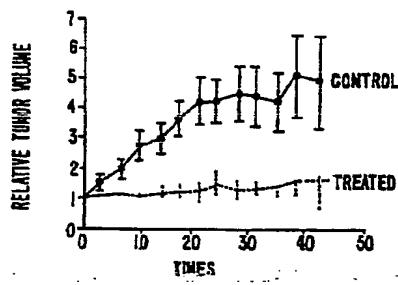


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

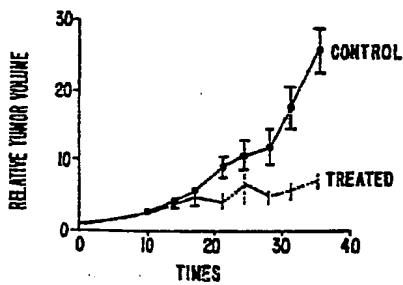
(51) International Patent Classification <sup>6</sup> :	A1	(11) International Publication Number: WO 99/48495 (43) International Publication Date: 30 September 1999 (30.09.99)
A61K 31/235		

(21) International Application Number:	PCT/US99/06220	(81) Designated States: AU, CA, IL, JP, KR, MX, ZA, European patent (AT, BE, CH, CY, DE, DK, ES, FL, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date:	22 March 1999 (22.03.99)	
(30) Priority Data:		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
60/079,313	25 March 1998 (25.03.98)	US
(71) Applicants:	BIOSOURCE TECHNOLOGIES, INC. [US/US]; 3333 Vaca Valley Parkway, Vacaville, CA 95688 (US). SRI INTERNATIONAL [US/US]; 333 Ravenswood Avenue, Menlo Park, CA 94025 (US).	
(72) Inventors:	TUSE, Daniel; 104 Elm Street, Menlo Park, CA 94025 (US). HIEBERT, Charles; Apartment 819, 1085 Tasman Drive, Sunnyvale, CA 94089 (US). LADERROUTE, Keith, R.; 461 Forest Avenue, Palo Alto, CA 94301 (US). WALEH, Nahid; 2344 Emerson Street, Palo Alto, CA 94301 (US).	
(74) Agents:	SNYDER, Joseph, R. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111-3834 (US).	

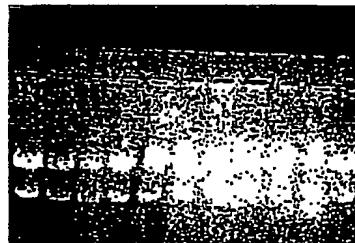
(54) Title: BENZOATES DERIVATIVES FOR INHIBITING ANGIOGENESIS



A



B



(57) Abstract

The present invention relates to methods for effectively inhibiting unwanted angiogenesis. More particularly, this invention relates to methods of treating diseases that show unwanted angiogenesis and to delivering anti-angiogenic activity to a mammal. In other aspects this invention relates to methods of reducing the level of tumor necrosis factor  $\alpha$ .

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**BENZOATES DERIVATIVES FOR INHIBITING ANGIOGENESIS**

5

**FIELD OF THE INVENTION**

The present invention relates to methods for effectively inhibiting  
10 unwanted angiogenesis. More particularly, this invention relates to methods of treating  
diseases associated with unwanted angiogenesis and to delivering anti-angiogenic activity  
to mammals having such diseases.

**BACKGROUND OF THE INVENTION**

15 Angiogenesis is the development of new blood vessels from existing  
microvessels. The process of generating new blood vessels plays an important role in  
embryonic development, in the inflammatory response, in the development of metastases  
(tumor induced angiogenesis or TIA), in diabetic retinopathy, in the formation of the  
arthritic panus and in psoriasis. Under normal physiological conditions, humans or  
20 animals only undergo angiogenesis in very specific, restricted situations. For example,  
angiogenesis is normally observed in wound healing, in fetal and embryonal development  
and in the formation of the corpus luteum, endometrium and placenta. The control of  
angiogenesis is a highly regulated system involving angiogenic stimulators and inhibitors.  
The control of angiogenesis has been found to be altered in certain disease states and, in  
25 many cases, the pathological damage associated with the disease is related to the  
uncontrolled angiogenesis.

In tumor angiogenesis, for example, capillary sprouts are formed, their  
formation being induced by a group of tumor cells. However, compared with blood  
vessels produced in normal angiogenic microenvironments, tumor microvessels are  
30 morphologically and functionally unique. Their vascular networks typically show  
disorganized or aberrant architecture, luminal sizes vary and blood flow can fluctuate  
chaotically. There are two principal types of tumor angiogenesis in terms of the events

which follow implantation of metastatic seedlings on surfaces and in organs. The first or primary angiogenesis is the initial vascularization of the mass of multiplying tumor cells and is regarded as an essential prerequisite for the survival and further growth of a metastatic deposit. The second is a continuing or secondary angiogenesis and is the 5 phenomenon which occurs in waves at the periphery of a growing tumor mass. This second angiogenesis is essential for the accretion of new microcirculatory territories into the service of the expanding and infiltrating tumor.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells and supports the 10 pathological damage seen in these conditions. The diverse pathological states created due to unregulated angiogenesis have been grouped together as angiogenic dependent or angiogenic associated diseases. Therapies directed to the control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

One example of a disease mediated by angiogenesis is ocular neovascular 15 disease. This disease is characterized by invasion of new blood vessels into the structures of the eye such as the retina or cornea. It is the most common cause of blindness and is involved in approximately twenty eye diseases. In age related macular degeneration, the associated visual problems are caused by an ingrowth of choroidal capillaries through defects in Bruch's membrane with proliferation of fibrovascular tissue beneath the retinal 20 pigment epithelium. Angiogenic damage is also associated with diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrothalamic fibroplasia. Diseases associated with retinal/choroidal neovascularization include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, 25 syphilis, pseudoxanthoma elasticum and Pagets disease.

Another disease in which angiogenesis is believed to be involved is 30 rheumatoid arthritis. The blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction.

An important area of current research in therapeutic oncology is focused 35 on the discovery and development of anti-angiogenic agents which target tumor vasculature by inhibiting or suppressing new blood vessel growth. Several kinds of compounds have been used to prevent angiogenesis. For instance, Taylor, *et al.* have used protamine to inhibit angiogenesis (*see, Taylor, et al., Nature* 297:307 (1982)).

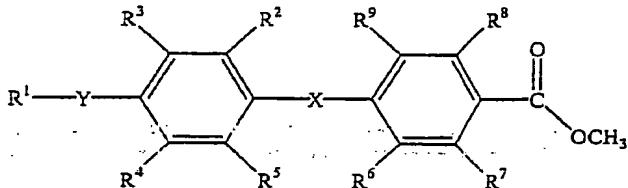
However, the toxicity of protamine limits its practical use as a therapeutic. In addition, Folkman, *et al.* have disclosed the use of heparin and steroids to control angiogenesis (see, Folkman, *et al.*, *Science* 221:719 (1983) and U.S. Pat. Nos. 5,001,116 and 4,994,443). Steroids, such as tetrahydrocortisol, which lack gluco- and mineral-corticoid activity, have been found to be angiogenic inhibitors. In addition, angiotatin proteins have been shown to reversibly inhibit proliferation of endothelial cells. Angiotatin is capable of inhibiting angiogenesis-related diseases and modulating angiogenic processes (see, e.g., WO 95/292420).

In view of the foregoing, it is apparent that there remains a need in the art for methods and compounds for inhibiting angiogenesis, either by competitively inhibiting an angiogenesis factor or by some other mechanism. Such methods and compounds would have an adverse effect on the growth of tumors and, in addition, could be used to treat many of the other diseases set forth above. The methods of the present invention fulfill this and other needs.

15

#### SUMMARY OF THE INVENTION

In one aspect, this invention relates to a method of inhibiting the vascularization of endothelial cells, the method comprising contacting a cell, tissue or organ which has endothelial cells with an anti-angiogenic amount of a compound of Formula I. Compounds of Formula I have the following general formula:



I

In Formula I, R<sup>1</sup> is a functional group including, but not limited to, C<sub>1</sub>-C<sub>6</sub>-alkyl.

In Formula I, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected and are functional groups including, but not limited to, hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>.

In Formula I, R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected and are functional groups including, but not limited to, hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>.

In Formula I, X, if present, is a functional group including, but not limited to, the following: oxygen, sulfur, -CH<sub>2</sub>-, or carboxy.

In Formula I, Y is a heteroatom including oxygen or sulfur.

5 In a preferred embodiment of the invention, the compound of Formula I is methyl 3,5-diiodo-4-(4'-methoxyphenoxy)benzoate ("BTO-956").

In another aspect, this invention relates to a method for effectively inhibiting unwanted angiogenesis in a tissue or organ, the method comprising contacting the cell with a compound of Formula I, or a pharmaceutical composition thereof, in an amount sufficient to inhibit angiogenesis. In a presently preferred embodiment, the cell is  
10 in a mammalian subject.

In yet another aspect, this invention relates to a method of treating mammalian diseases mediated by or associated with undesired and uncontrolled angiogenesis, the method comprising administering to a mammal an anti-angiogenic compound of Formula I in a dosage sufficient to inhibit angiogenesis. These methods are  
15 useful for ameliorating the effects of conditions that are characterized by abnormal or undesirable angiogenesis or endothelial cell proliferation.

In another aspect, this invention relates to methods that reduce the level of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) produced by a cell.

20 In still yet another aspect, this invention relates to methods of using such compounds to reduce TNF- $\alpha$  production and to treat inflammatory diseases.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 FIG. 1 illustrates the mean growth response as relative tumor volume of MDA MB-231 (A) and OVCAR3 (B) tumors in nude mice exposed to BTO-956.

FIG. 2 illustrates that BTO-956 induces a prometaphase arrest in MCF-7 breast carcinoma cells.

30 FIG. 3 illustrates the inhibition of *in vitro* microtubules assembly by BTO-956.

FIG. 4 illustrates the disruption of microtubule networks by BTO-956.

FIG. 5 illustrates that BTO-956 competes with colchicine for binding to tubulin *in vitro*.

35 FIG. 6 illustrates that BTO-956 inhibits the proliferation of human vascular endothelial cells.

FIG. 7 illustrates that BTO-956 reduces TNF- $\alpha$  mRNA accumulation.

**FIG. 8** illustrates the effect of different concentrations of BTO-956 on TNF- $\alpha$  gene expressions.

**FIG. 9** illustrates that total RNA was intact in cells treated with different concentrations of BTO-956 and colchicine.

## DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

### A. DEFINITIONS

The term "angiogenesis" refers to the generation of new blood vessels into 10 cells, tissue, organs or tumors.

The term "metastasis" refers to the process by which tumor cells are spread to distant parts of the body. The term is also used herein to refer to a tumor that develops through the metastatic process.

The term "independently selected" is used herein to indicate that the R 15 groups, e.g., R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup>, can be identical or different (e.g., R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> may all be hydrogens or R<sup>1</sup> and R<sup>4</sup> may be hydrogen and R<sup>2</sup> and R<sup>3</sup> may be halogen, etc.).

The term "alkyl" is used herein to refer to a branched or unbranched, 20 saturated or unsaturated, monovalent hydrocarbon radical having from 1-12 carbons and preferably, from 1-6 carbons. When the alkyl group has from 1-6 carbon atoms, it is referred to as a "lower alkyl." Suitable alkyl radicals include, for example, methyl, ethyl, n-propyl, i-propyl, 2-propenyl (or allyl), n-butyl, t-butyl, i-butyl (or 2-methylpropyl), etc. As used herein, the term encompasses "substituted alkyls."

"Substituted alkyl" refers to alkyl as just described including one or more 25 functional groups such as lower alkyl, aryl, acyl, halogen (i.e., alkylhalos, e.g., CF<sub>3</sub>), hydroxy, amino, alkoxy, alkylamine, acylamino, acyloxy, aryloxy, aryloxyalkyl, mercapto, both saturated and unsaturated cyclic hydrocarbons, heterocycles and the like. These groups may be attached to any carbon of the alkyl moiety.

The term "S-alkyl" is used herein to refer to the group -SR, where R is lower alkyl or substituted lower alkyl as defined herein.

The term "aryl" is used herein to refer to an aromatic substituent which 30 may be a single aromatic ring or multiple aromatic rings which are fused together, linked covalently, or linked to a common group such as a methylene or ethylene moiety. The common linking group may also be a carbonyl as in benzophenone. The aromatic ring(s) may include phenyl, naphthyl, biphenyl, diphenylmethyl and benzophenone among others. The term "aryl" encompasses "arylalkyl."

"Substituted aryl" refers to aryl as just described including one or more functional groups such as lower alkyl, acyl, halogen, alkylhalos (e.g. CF<sub>3</sub>), hydroxy, amino, alkoxy, alkylamine, acylamino, acyloxy, mercapto and both saturated and unsaturated cyclic hydrocarbons which are fused to the aromatic ring(s), linked covalently or linked to a common group such as a methylene or ethylene moiety. The linking group may also be a carbonyl such as in cyclohexyl phenyl ketone. The term "substituted aryl" encompasses "substituted arylalkyl."

The term "halogen" is used herein to refer to fluorine, bromine, chlorine and iodine atoms.

10 The term "hydroxy" is used herein to refer to the group —OH.

The term "amino" is used to refer to the group —NRR', where R and R' may independently be hydrogen, alkyl, substituted alkyl, aryl, substituted aryl or acyl.

The term "nitro" is used herein to refer to the group —NO<sub>2</sub>.

15 The term "alkoxy" is used herein to refer to the —OR group, where R is a lower alkyl, substituted lower alkyl, aryl, substituted aryl, arylalkyl or substituted arylalkyl wherein the alkyl, aryl, substituted aryl, arylalkyl and substituted arylalkyl groups are as described herein. Suitable alkoxy radicals include, for example, methoxy, ethoxy, phenoxy, substituted phenoxy, benzyloxy, phenethyloxy, t-butoxy, etc.

20 The term "alkenyl" is used herein to refer to an unsaturated branched, straight chain or cyclic monovalent hydrocarbon radical having at least one carbon-carbon double bonds. The radical can be in either the *cis* or *trans* conformation about the double bond(s). Suitable alkenyl radicals include, for example, ethenyl, propenyl, isopropenyl, cyclopropenyl, butenyl, isobut enyl, cyclobutenyl, *tert*-butenyl, pentenyl, hexenyl, etc.

25 The term "alkynyl" is used herein to refer to an unsaturated branched, straight chain or cyclic monovalent hydrocarbon radical having at least one carbon-carbon triple bond. Suitable alkynyl radicals include, for example, ethynyl, propynyl, butynyl, isobutynyl, pentynyl, hexynyl, etc.

30 The term "contacting" is used herein interchangeably with the following: combined with, added to, mixed with, passed over, incubated with, flowed over, etc. Moreover, the compounds of present invention can be "administered" by any conventional method such as, for example, parenteral, oral, topical and inhalation routes as described herein.

35 The term "pharmaceutically acceptable salt" refers to those salts of compounds which retain the biological effectiveness and properties of the free bases and which are obtained by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid,

ethanesulfonic acid, ptoluenesulfonic acid, salicylic acid and the like. Pharmaceutically acceptable salts include, for example, alkali metal salts, such as sodium and potassium, alkaline earth salts and ammonium salts.

"An amount sufficient," "an effective amount," "therapeutically effective amount" or "anti-angiogenic" amount refer to an amount of a compound or composition effective to depress, suppress or inhibit angiogenesis or result in amelioration of symptoms associated with an angiogenic disease. The desired result can be either a subjective relief of a symptom(s) or an objectively identifiable improvement in the recipient of the dosage, a decrease in the vascularization of endothelial cells or a decrease 10 in the rate of angiogenesis as noted by a clinician or other qualified observer.

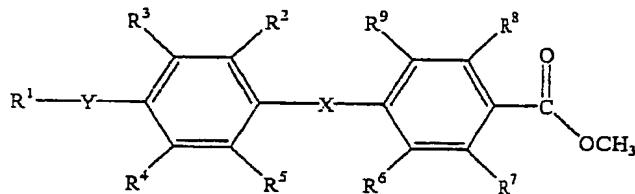
The terms "treating cancer," "therapy," and the like refer generally to any improvement in the mammal having the cancer wherein the improvement can be ascribed to treatment with the compounds of the present invention. The improvement can be either subjective or objective. For example, if the mammal is human, the patient may 15 note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Alternatively, the clinician may notice decrease in tumor size or tumor burden based on physical exam, laboratory parameters, tumor markers or radiographic findings. Some laboratory signs that the clinician may observe for response to therapy include normalization of tests such as white blood cell count, red blood cell 20 count, platelet count, erythrocyte sedimentation rate, and various enzyme levels. Additionally, the clinician may observe a decrease in a detectable tumor marker. Alternatively, other tests can be used to evaluate objective improvement such as sonograms, nuclear magnetic resonance testing and positron emissions testing.

"Inhibiting the growth of tumor cells" can be evaluated by any accepted 25 method of measuring whether growth of the tumor cells has been slowed or diminished. This includes direct observation and indirect evaluation such as subjective symptoms or objective signs as discussed above.

## B. THE COMPOUNDS

The present invention relates to the discovery that compounds of Formula I are useful for inhibiting angiogenesis and/or for treating angiogenic diseases. Compounds of Formula I have the following general formula:

5



I

wherein R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup>, R<sup>9</sup>, X and Y are as defined above. In a preferred embodiment of the invention, the compound of Formula I is methyl 3,5-diido-4-(4'-methoxyphenoxy)benzoate ("BTO-956").

10

The compounds used in the methods of the current invention can be made in accordance with the procedure outlined in Borrow, *et al.*, *J. Chem. Soc.* 1949, S185-190 and WO 97/46228, the teachings of which are incorporated herein by reference. In general, the process is accomplished in a series of steps. For instance, starting with the case of BTO-956, a substituted phenol and a substituted benzoate are reacted to yield 15 methyl 3,5-dinitro-4-(4-methoxyphenoxy)benzoate. The nitro groups of the benzoate are then reduced to amines and subsequently replaced by iodine. This method for preparing BTO-956 is essentially as described in: Masuda, K., Imashiro, Y., and Okada, Y. "Synthesis of Triiodothyroformic Acid and its Derivatives," *J. Takeda Res. Lab.*, 1970, 29, 545-552, the teachings of which are incorporated herein by reference. Further 20 synthetic details are set forth in Example 1.

Compounds suitable for use in the methods of the present invention can readily be identified using *in vitro* and *in vivo* screening assays. Such assays may screen for the ability of a particular compound to inhibit angiogenesis or the vascularization of endothelial cells *in vitro* and *in vivo*. For instance, the chick embryo chorioallantoic 25 membrane (CAM) assay, which is described in more detail below, can be used to screen a given compound for its ability to inhibit vascularization. In the chorioallantoic membrane assay, fertilized chick embryos are removed from their shell on day 3 or 4, and a methylcellulose disc containing a compound of Formula I is implanted on the chorioallantoic membrane. The embryos are examined 48 hours later and, if a clear 30 avascular zone appears around the methylcellulose disc, the diameter of that zone is measured. This assay can be used to assess the anti-angiogenic properties of the compounds of Formula I.

Another useful screening assay to assess the efficacy of compounds of Formula I is the corneal micropocket angiogenesis assay (CMA). The rat corneal micropocket assay can be used to assess the ability of compounds of Formula I to inhibit corneal angiogenesis (see, "Quantitative Angiogenesis Assays: Progress and Problems," 5 *Nat. Med.*, 3: 1203-1208, 1997) and "Inhibition of Tumor Angiogenesis Using a Soluble Receptor Establishes a Role for Tie2 in Pathologic Vascular Growth," *J. Clin. Invest.*, 100: 2072-2078, 1997). In this assay, the compound of Formula I is mixed with a polymer (e.g., Hydron solution; Interferon Sciences, New Brunswick, NJ) and implanted in a small pocket surgically created in the superficial layers of the cornea of a rat. Under 10 normal circumstances, this wound stimulates an angiogenic response which is readily visible as the appearance of neovessels on the normally avascular cornea. If the compound of Formula I is effective, specifically as an anti-angiogenic agent, it inhibits or blocks this response. In one experimental design, a group of five animals (including a control group with only polymer implants) is tested over a range of drug doses which can 15 induce tumor growth delay. Three doses are tested in the assay. Assessment of an anti-angiogenic response by this method is categorical. In other words, a treated eye is either positive or negative for corneal angiogenesis. This assay determines whether a compound of Formula I is directly anti-angiogenic in an *in vivo* mammalian model of angiogenesis.

20 In addition, the human microvascular endothelial cell assay (HMVEC) can be used to assess the efficacy of compounds of Formula I. HMVEC are seeded into a 96-well plate at a x concentration of  $5 \times 10^3$  cells/well in a volume of 100  $\mu\text{l}$ /well of Endothelial Growth Medium. Plates are then incubated at 37°C in 5% CO<sub>2</sub> for 24 h and then aliquots of the compound of Formula I are added to the HMVEC preparations and 25 plates are then incubated at 37°C in 5% CO<sub>2</sub> for 3 days. The relative number of cells is determined by adding 20  $\mu\text{l}/\text{ml}$  of Alamar Blue for 3-6 h at 37°C and measuring color changes indicating metabolic activity by using a Fluorescence Measurement System. In this assay, the intensity of the fluorophore signal is directly proportional to cell number.

30 The HMVEC assay can also be carried out using human umbilical vein microvascular endothelial cells (HUMVEC). This assay is carried out similarly to the above assay, but HUMVEC cells are used. In addition, other assays known to those of skill in the art can readily be used to screen the compounds of the present invention for anti-angiogenic properties.

35 It will be readily apparent to those skilled in the art that the compounds of Formula I can be administered alone, in the form of a pharmaceutically acceptable salt and/or in the form of a pharmaceutical composition.

**C. USES FOR THE COMPOUNDS OF THE PRESENT INVENTION**

As explained above, the present invention relates to the discovery that the compounds of Formula I are useful for inhibiting angiogenesis and, in turn, for treating diseases associated with unwanted angiogenesis. As such, in one embodiment, the 5 present invention provides a method of inhibiting unwanted angiogenesis in a cell, the method comprising contacting the cell with an effective amount, *i.e.*, an anti-angiogenic amount, of a compound of Formula I. In another embodiment, the present invention provides a method of inhibiting the vascularization of endothelial cells, the method comprising contacting a cell, tissue or organ containing the endothelial cells with an 10 effective amount of a compound of Formula I. In a presently preferred embodiment, the cells are in a mammalian subject.

This invention relates to a method of treating mammalian diseases associated with undesired and uncontrolled angiogenesis, the method comprising administering to a mammal an anti-angiogenic compound of Formula I in an amount, *i.e.*, 15 a dosage, sufficient to inhibit angiogenesis. The particular dosage of a compound of Formula I required to inhibit angiogenesis and/or angiogenic diseases will depend upon the severity of the condition, the route of administration, and related factors that will be decided by the attending physician. Generally, accepted and effective daily doses will be the amount sufficient to effectively inhibit angiogenesis and/or angiogenic diseases.

20 The methods of treatment provided by this invention are practiced by administering to a mammal in need thereof a dose of a compound of Formula I (or a pharmaceutically acceptable salt or solvate thereof) that is effective to Inhibit angiogenesis and/or angiogenic diseases. The term "inhibit" is used herein to include its generally accepted meaning which includes prophylactically treating a human subject to 25 incurring angiogenesis and/or angiogenic diseases, and holding in check and/or treating existing angiogenesis and/or angiogenic diseases. As such, the present invention includes both medical therapeutic and/or prophylactic treatment, as appropriate.

The methods of the present invention can be used to treat a wide variety of diseases. Diseases associated with corneal neovascularization that can be treated using 30 the methods of the present invention include, but are not limited to, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrobulbar fibroplasia, epidemic keratoconjunctivitis, vitamin A deficiency, contact lens overwear, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, sjogrens, acne rosacea, phlyctenulosis, syphilis, Mycobacteria infections, lipid degeneration, chemical burns, 35 bacterial ulcers, fungal ulcers, Herpes simplex infections, Herpes zoster infections, protozoan infections, Kaposi sarcoma, Mooren ulcer, Terrien's marginal degeneration, marginal keratolysis, trauma, rheumatoid arthritis, systemic lupus, polyarteritis, Wegeners

sarcoidosis, Scleritis, Steven's Johnson disease, periphigoid radial keratotomy, and corneal graft rejection.

Diseases associated with retinal/choroidal neovascularization that can be treated using the methods of the present invention include, but are not limited to, diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Pagets disease, vein occlusion, artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales disease, Bechets disease, infections causing a retinitis or choroiditis, presumed ocular histoplasmosis, Bests disease, myopia, optic pits, Stargarts disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma and post-laser complications. Other diseases include, but are not limited to, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue, including all forms of proliferative vitreoretinopathy, whether or not associated with diabetes.

Diseases associated with chronic inflammation can also be treated using the methods of the present invention. Diseases with symptoms of chronic inflammation include, but are not limited to, inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, psoriasis, sarcoidosis and rheumatoid arthritis. Unwanted or uncontrolled angiogenesis is a key element that these chronic inflammatory diseases all have in common. The chronic inflammation depends on continuous formation of capillary sprouts to maintain an influx of inflammatory cells. The influx and presence of the inflammatory cells produce granulomas and, thus, maintain the chronic inflammatory state. Inhibition of angiogenesis using the compositions and methods of the present invention prevents the formation of the granulomas, thereby alleviating the disease.

As mentioned above, the methods of the present invention can be used to treat patients with inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis. Crohn's disease occurs as a chronic transmural inflammatory disease that most commonly affects the distal ileum and colon, but may also occur in any part of the gastrointestinal tract from the mouth to the anus and perianal area. Patients with Crohn's disease generally have chronic diarrhea associated with abdominal pain, fever, anorexia, weight loss and abdominal swelling. Prevention of angiogenesis by the compositions and methods of the present invention inhibits the formation of the sprouts and prevents the formation of granulomas. Ulcerative colitis is also a chronic, nonspecific, inflammatory and ulcerative disease arising in the colonic mucosa and is characterized by the presence of bloody diarrhea.

The inflammatory bowel diseases also exhibit extra intestinal manifestations, such as skin lesions. Such lesions are characterized by inflammation and angiogenesis and can occur at many sites other than in the gastrointestinal tract. The compositions and methods of the present invention can also be used to treat these lesions 5 by preventing the angiogenesis, thus reducing the influx of inflammatory cells and the lesion formation.

Sarcoidosis is another chronic inflammatory disease that is characterized as a multisystem granulomatous disorder. The granulomas of this disease can form anywhere in the body and, thus, the symptoms depend on the site of the granulomas and 10 whether the disease active. The granulomas are created by the angiogenic capillary sprouts providing a constant supply of inflammatory cells. The compounds and method of this invention can be used to treat sarcoidosis.

The methods of the present invention can also be used to treat the chronic inflammatory conditions associated with psoriasis. Psoriasis, a skin disease, is another 15 chronic and recurrent disease that is characterized by papules and plaques of various sizes. Prevention of the formation of the new blood vessels necessary to maintain the characteristic lesions leads to relief from the symptoms.

Another disease which can be treated using the methods of the present invention is rheumatoid arthritis. Rheumatoid arthritis is a chronic inflammatory disease 20 characterized by nonspecific inflammation of the peripheral joints. It is thought that the blood vessels in the synovial lining of the joints undergo angiogenesis. In addition to forming new vascular networks, the endothelial cells release factors and reactive oxygen species that lead to pannus growth and cartilage destruction. The factors involved in angiogenesis may actively contribute to, and help maintain, the chronically inflamed state 25 of rheumatoid arthritis.

Other diseases that can be treated using the methods of the present invention are hemangiomas, Osler-Weber-Rendu disease, or hereditary hemorrhagic telangiectasia, solid or blood-borne tumors and acquired immune deficiency syndrome.

The methods of this invention are also effective in inhibiting angiogenesis 30 associated with malignant tumor growth. This includes cancerous tumor growth on cells tissues and organs. The methods of the present invention are useful in treating the growth of a number of tumor cells and for treating a wide variety of cancers. Such tumor cells include, by way of example and not limitation, lung, colon, breast, ovarian, prostate and hepatic tumor cells as well as squamous cell carcinomas. Such cancers include, by 35 way of example and not limitation, carcinomas such as pharynx, colon, rectal, pancreatic, stomach, liver, lung, breast, skin, prostate, ovary, cervical, uterine and bladder cancers; leukemias; lymphomas; gliomas; retinoblastomas; and sarcomas.

In a particularly preferred embodiment, the present invention relates to methods of administering compounds of Formula I in combination with active immunotherapy (e.g., tumor vaccination). Because the compounds of Formula I are not immunotoxic, the immune system is not significantly suppressed and, thus, active 5 immunotherapy can advantageously be carried out in combination with the chemotherapy. When used in conjunction with immunotherapy, the compound of Formula I can be administered prior to and/or during administration of the immunotherapeutic agent (e.g., a tumor vaccine).

In still another embodiment, the present invention provides a method for 10 reducing the level of TNF- $\alpha$  produced by a cell. TNF- $\alpha$  and its various modes of action are generally described by Abbas, *et al.*, *Cellular and Molecular Immunology*, Abbas, *et al.*, 2nd Ed., W.B. Saunders Company, 1994, pp. 244-249, the teachings of which are incorporated herein by reference. TNF- $\alpha$  plays an integral role in destroying tumors, mediating responses to tissue injury and protecting hosts from infections by 15 various microorganisms. However, its activity appears to be excessive in some disease states and inflammatory reactions such as rheumatoid arthritis, cachexia and septic shock. The excess TNF- $\alpha$  results in an exaggerated immune response exemplified by over stimulation of interleukin-6 and granulocyte/macrophage-colony stimulating factor (GM-CSF) secretion, enhanced cytotoxicity of polymorphonuclear neutrophils and prolonged 20 expression of cellular adhesion molecules, all of which can have detrimental effects.

Contacting cells with the compounds of Formula I results in decreased levels of TNF- $\alpha$ . Reduced levels of TNF- $\alpha$  can result from any of several mechanisms including, for example, downregulation of expression of a gene that encodes TNF- $\alpha$ , a reduction in TNF- $\alpha$  mRNA stability or translation efficiency, decreased stability of the 25 TNF- $\alpha$  polypeptide, and reduced secretion of TNF- $\alpha$  from a cell. Reduced levels of TNF- $\alpha$  can be measured in a cell, biological sample or the blood stream. As a result of their ability to inhibit TNF- $\alpha$ , the compounds of Formula I can be used to treat inflammatory diseases. Such diseases include, but are not limited to, the inflammatory diseases set forth above (e.g., chronic inflammation, chronic disease, inflammatory bowel disease, 30 sarcoidosis, psoriasis, rheumatoid arthritis, and the like). Using the assay set forth in Example VIII, compounds of Formula I can readily be screened for their ability to reduce TNF- $\alpha$  levels.

TNF- $\alpha$  is noted for its pro-inflammatory actions which result in tissue injury, such as induction of procoagulant activity on vascular endothelial cells, increased 35 adherence of neutrophils and lymphocytes, and stimulation of the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells. As such, targeting moieties which are directed to these cells and which are conjugated to liposomes

or other drug delivery systems comprising the compounds of Formula I are preferred embodiments of this invention. For instance, in a preferred embodiment, monoclonal antibodies to TNF- $\alpha$  (Tracey, et al., *Nature* 1987, 330, 662-664; Silva, et al., *J. Infect. Sis.* 1990, 162, 421-427; and Williams, et al., *Proc. Natl. Acad. Sci.* 1992, 89, 9784-9788) are conjugated to liposomes comprising compounds of Formula I.

5 Moreover, in accordance with the above methods, mammalian subjects include, but are not limited to, humans, laboratory animals, domestic pets and farm animals.

10 D. PHARMACEUTICAL FORMULATIONS/ROUTES OF ADMINISTRATION

In the methods of the present invention, the compounds of Formula I can be delivered or administered to a mammal, e.g., a human patient, alone, in the form of a pharmaceutically acceptable salt, or in the form of a pharmaceutical composition where 15 the compound is mixed with suitable carriers or excipient(s) in a therapeutically effective amount, e.g., at doses effective to depress, suppress or inhibit angiogenesis or result in amelioration of symptoms associated with angiogenic diseases.

The compounds of Formula I, which are used in the methods of the present invention, can be incorporated into a variety of formulations for therapeutic 20 administration. More particularly, compounds of Formula I can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, pills, powders, granules, dragees, gels, slurries, ointments, solutions, suppositories, injections, inhalants and aerosols. As 25 such, administration of the compounds can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, etc., administration. Moreover, the compound can be administered in a local rather than systemic manner, for example via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation. In addition, the compounds can be 30 administered in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. Such liposomes will be targeted to and taken up selectively by the tumor.

In addition, the compounds of Formula I can be formulated with common excipients, diluents or carriers, and compressed into tablets, or formulated as elixirs or 35 solutions for convenient oral administration, or administered by the intramuscular or intravenous routes. The compounds can be administered transdermally, and may be formulated as sustained release dosage forms and the like.

Compounds of Formula I can be administered alone, in combination with each other, or they can be used in combination with other known compounds (e.g., other anti-cancer drugs or other drugs, such as AZT, anti-inflammatories, antibiotics, corticosteroids, vitamins, etc.). For instance, the compound of Formula I can be used in conjunctive therapy with other known anti-angiogenic chemotherapeutic or antineoplastic agents (e.g., vinca alkaloids, antibiotics, antimetabolites, platinum coordination complexes, etc.). For instance, the compounds of Formula I can be used in conjunctive therapy with a vinca alkaloid compound, such as vinblastine, vincristine, taxol, etc.; an antibiotic, such as adriamycin (doxorubicin), dactinomycin (actinomycin D), daunorubicin (daunomycin, rubidomycin), bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin C), etc.; an antimetabolite, such as methotrexate, cytarabine (AraC), azauridine, azaribine, fluorodeoxyuridine, deoxycoformycin, mercaptopurine, etc.; or a platinum coordination complex, such as cisplatin (cis-DDP), carboplatin, etc. In addition, those of skill in the art will appreciate that the compounds of the present invention can be used in conjunctive therapy with other known anti-angiogenic chemotherapeutic or antineoplastic compounds. In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination with other pharmaceutically active compounds.

Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences* (Mack Publishing Company, Philadelphia, PA, 17th ed. (1985)), which is incorporated herein by reference. Moreover, for a brief review of methods for drug delivery, see, Langer, *Science* 249:1527-1533 (1990), which is incorporated herein by reference. The pharmaceutical compositions described herein can be manufactured in a manner that is known to those of skill in the art, i.e., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. The following methods and excipients are merely exemplary and are in no way limiting.

For injection, the compounds can be formulated into preparations by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives. Preferably, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration,

penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds of Formula I can be formulated readily by combining with pharmaceutically acceptable carriers that are well known in the art. Such carriers enable the compounds to be formulated as tablets, pills, dragees, capsules, emulsions, lipophilic and hydrophilic suspensions, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by mixing the compounds with a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after 5 adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If 10 desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer 20 solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as 25 talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All 30 formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon 35 dioxide or other suitable gas, or from propellant-free, dry-powder inhalers. In the case of

a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

5       The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulator agents such as  
10 suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic  
15 fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active  
20 ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter, carbowaxes, polyethylene glycols or other glycerides, all of which melt at  
25 body temperature, yet are solidified at room temperature.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long-acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by  
30 intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of  
35 delivery vehicles or carriers for hydrophobic drugs. In a presently preferred embodiment, long-circulating, i.e., stealth, liposomes are employed. Such liposomes are generally

described in Woodle, *et al.*, U.S. Patent No. 5,013,556, the teaching of which is hereby incorporated by reference.

Monoclonal antibodies optionally conjugated to liposomes and directed against a tumor marker, TNF- $\alpha$ , or a TNF- $\alpha$  receptor, is another strategy that can be employed. In addition, targeting of a marker on abnormal tumor vasculature can be employed. The targeting moiety when coupled to a toxic drug or radioisotope will act to concentrate the drug where it is needed. Ligands for tumor-associated vessel markers can also be used. For example, a cell adhesion molecule that binds to a tumor vascular element surface marker can be employed. Liposomes and other drug delivery systems can also be used, especially if their surface contains a ligand to direct the carrier preferentially to the tumor vasculature. Liposomes offer the added advantage of shielding the drug from most normal tissues, thereby reducing the inherent toxicity of many compounds. When coated with polyethylene glycol (PEG) (*i.e.*, stealth liposomes) to minimize uptake by phagocytes and with a tumor vasculature-specific targeting moiety, liposomes offer longer plasma half-lives, lower non-target tissue toxicity, and increased efficacy over non-targeted drug. Other targeting strategies include, but are not limited to, ADEPT (antibody-directed enzyme prodrug therapy), GDEPT (gene-directed EPT) and VDEPT (virus-directed EPT). In ADEPT, the targeting of an inactive prodrug to a tumor mass is effected by an antibody against a tumor-associated marker. The enzyme milieu in or about the tumor transforms the prodrug into an active toxic agent that then acts on the tumor tissue. Similarly, differential gene expression or viral targeting at the tumor site is used to activate a prodrug into its active, toxic form in GDEPT and VDEPT, respectively. Other strategies include targeting differentially expressed genes, enzymes or surface markers that appear on tumor-associated vasculature, to effect control of tumor growth. Using the foregoing methods, the compounds of Formula I can be targeted to the tumor vasculature to effect control of tumor progression or to other sites of interest (*e.g.*, endothelial cells).

Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various types of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not

limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in a therapeutically effective amount. The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician. Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, a therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC<sub>50</sub> as determined in cell culture (*i.e.*, the concentration of test compound that is lethal to 50% of a cell culture), or the IC<sub>100</sub> as determined in cell culture (*i.e.*, the concentration of compound that is lethal to 100% of a cell culture). Such information can be used to more accurately determine useful doses in humans. Initial dosages can also be estimated from *in vivo* data.

Moreover, toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, by determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effect is the therapeutic index and can be expressed as the ratio between LD<sub>50</sub> and ED<sub>50</sub>. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a dosage range that is not toxic for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.,* Fingl *et al.*, 1975, In: *The Pharmacological Basis of Therapeutics*, Ch. 1, p. 1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound which are sufficient to maintain therapeutic effect. Usual patient dosages for oral administration range from about 50-2000 mg/kg/day, commonly from about 100-1000 mg/kg/day, preferably from about 150-700 mg/kg/day and most preferably from about 250-500 mg/kg/day. Preferably, therapeutically effective serum levels will be achieved by administering multiple doses each day. In cases of local

administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

5      F.      EXAMPLES

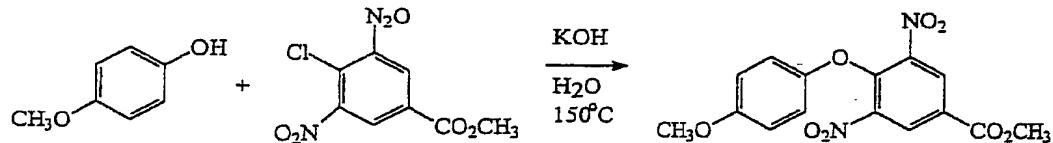
The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield  
10     essentially the same results.

EXAMPLE I

This example illustrates the synthesis of methyl 3,5-diodo-4-(4-methoxyphenoxy)benzoate (BTO-956).

15     The synthesis of BTO-956 is accomplished in a series of steps, first yielding methyl 3,5-dinitro-4-(4-methoxyphenoxy)benzoate, the nitro groups of which are then reduced to amines and subsequently replaced by iodine. The method for preparing BTO-956 is essentially as described in: Masuda, K., Imashiro, Y., and Okada, Y.  
20     Synthesis of triiodothyroformic acid and its derivatives. *J. Takeda Res. Lab.* 1970, 29, 545-552.

1.      Methyl 3,5-dinitro-4-(4-methoxyphenoxy)benzoate

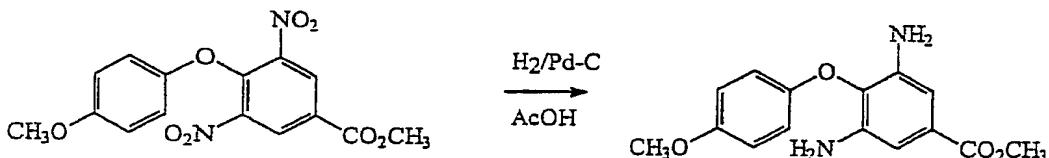


25	Methyl 3,5-dinitro-4-chlorobenzoate 4-Methoxyphenol Potassium hydroxide Water	20.2 g; 77.5 mmol 10.0 g; 80.6 mmol 4.7 g; 82.0 mmol 20 mL; solvent
----	--	--

To a 100 mL round-bottomed flask containing potassium hydroxide (4.7 g;  
30     82.0 mmol) dissolved in water (20 mL) was successively added 4-methoxyphenol (10.0 g;  
80.6 mmol) and methyl 4-chloro-3,5-dinitrobenzoate (20.2 g; 77.5 mmol). The flask was fitted with a reflux condenser and the reaction heated at 150°C (oil bath) for 3 hours. After cooling to room temp, the reaction mixture was transferred to a large mortar and

triturated with cold 2N NaOH (100 mL) to remove unreacted phenol. The solid was collected by filtration and air-dried to give 21.5 g of crude product. Crystallization from absolute ethanol gave 17.7 g (65.6 %) of pure methyl 3,5-dinitro-4-(4-methoxyphenoxy)benzoate as light yellow needles. 300 MHz <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 3.77 (s, 3H, OCH<sub>3</sub>), 4.02 (s, 3H, OCH<sub>3</sub>), 6.82 (m, 4H, ArH), 8.70 (s, 2H, ArH).

2. Methyl 3,5-diamino-4-(4-methoxyphenoxy)benzoate



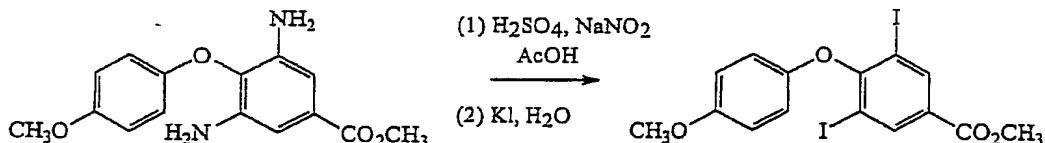
M.W. 348.27

M.W. 288.27

10 Methyl 3,5-dinitro-4-(4-methoxyphenoxy)benzoate      20.2 g; 77.5 mmol  
10% palladium on charcoal      0.7 g; catalyst  
Glacial acetic acid      80 mL; solvent

To a Parr shaker bottle containing a suspension of methyl 3,5-di-nitro-4-(4-methoxyphenoxy)-benzoate (20.2 g; 77.5 mmol) in glacial acetic acid (80 mL) was added 10% palladium on charcoal (0.7 g). The bottle was shaken under an atmosphere of hydrogen (3 atm) until no more hydrogen was consumed. The catalyst was filtered off and the resulting solution concentrated to approximately 10 mL. The residue was dissolved in acetone (50 mL) and heated on a steam bath while water (100 mL) was added in portions. Upon cooling, medium brown needles formed which were collected by suction filtration and dried to give 7.1 g (86%) of methyl 3,5-diamino-4-(4-methoxyphenoxy)benzoate. 300 MHz <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 3.73 (s, 3H, OCH<sub>3</sub>), 3.80 (bs, 4H, ArNH<sub>2</sub>) 3.86 (s, 3H, CCH<sub>3</sub>), 5.84 (m, 4H, ArH), 6.91 (s, 2H, ArH).

25 3. Methyl 3,5-diodo-4-(4-methoxyphenoxy)benzoate



M.W. 288.27

M.W. 510.06

	Methyl 3,5-diamino-4-(4-methoxyphenoxy)benzoate	4.3 g; 14.2 mmol
	Sodium nitrite	2.6 g; 37.4 mmol
	Glacial acetic acid	80 mL; solvent
	Sulfuric acid	26 mL; solvent
5	Potassium iodide	20 g; 120.0 mmol
	Water	30 mL; solvent

Sulfuric acid (26 mL) was placed in a three-necked flask equipped with a mechanical stirrer and cooled in an ice bath. Sodium nitrite (2.58 g, 37.4 mmol) was added in small portions, and the mixture was stirred for 20 min to form a thick solution.

10 To this was added a slurry of methyl 3,5-diamino-4-(4-methoxyphenoxy)benzoate (4.30 g, 14.22 mmol) in glacial acetic acid (80 mL), dropwise over a period of 30 min, keeping the temperature below 10°C with the ice bath. The reddish brown solution was stirred at below 10°C for 45 min, after which it was poured slowly into an aqueous (30 mL) solution of potassium iodide (20 g) at room temperature, with vigorous stirring. A thick

15 suspension formed, and was allowed to stir at RT for 1h. The reaction mixture was then heated in an oil-bath to 80°C (internal temperature) for 15 min, and then allowed to cool to RT. The solution was filtered and the black gummy residue was dissolved in 300 mL of acetone. The dark filtrate, when refrigerated overnight, deposited a dark residue, which was collected by decanting the supernatant, and the residue was dissolved in 100

20 mL of acetone. The combined acetone solution was filtered over a pad of basic alumina (5 cm) in a 150 mL sintered glass funnel to remove some colored impurities. The alumina pad was washed with 100 mL acetone and the red filtrate was evaporated to dryness, to give a dark solid as crude product. This was purified by flash chromatography on silica gel, eluting with hexanes: CH<sub>2</sub>Cl<sub>2</sub> (60:40). Initial fractions containing the pure

25 product were pooled and evaporated to yield 1.67 g of the desired product as an off-white solid. This compound gave a single, clean spot on TLC (Hexanes: CH<sub>2</sub>Cl<sub>2</sub>; 1:1; R<sub>f</sub> 0.35). Impure fractions were pooled and triturated with absolute EtOH for 16 hours at room temperature. The solid was filtered and dried to yield another 0.3 g of the product as a cream solid, which contained ~5% of the slow-moving impurity as evidenced by TLC (R<sub>f</sub> 0.29).

30 Total yield of the product was 27%. 300 MHz <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 3.78 (s, 3H, OCH<sub>3</sub>), 3.94 (s, 3H, COOCH<sub>3</sub>), 6.70 and 6.83 (two d, AA'XX', 4H, p-subs. Ar-H), 8.51 (s, 2H, Ar-H).

#### EXAMPLE II

35 This example illustrates the anti-angiogenic properties of methyl 3,5-diiodo-4-(4-methoxyphenoxy)benzoate in the chick chorioallantoic membrane assay (CAM). The endpoint of the CAM assay was a quantitative determination of basement membrane biosynthesis by measuring the incorporation of <sup>14</sup>C-proline into Type IV collagenous protein.

A. Approach

The CAM assay involves the development of live chick embryos in Petri dishes under special sterile conditions. Therefore, only limited numbers of embryos can be used for evaluation of compounds in a single experiment. For this reason, two separate assays were conducted to test the three Biosource compounds at three concentrations per compound. In this assay, a known angiogenesis inhibitor, 2-methoxyestradiol (2-ME), was used as the positive control, and human fibroblast growth factor (hFGF) was used to induce angiogenesis in the CAM.

10

B. Materials

Fertilized eggs were supplied by Melody Ranch, Aptos, CA. L-[U-<sup>14</sup>C] proline (specific activity, 290 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Collagenase and 2-ME were obtained from Sigma Chemical Co., St. Louis, MO. Silicone ring cups were obtained by cutting silicone tubing (3 mm diameter) into small "O" rings 1 mm in thickness. These silicone ring cups can be reused many times if they are sterilized prior to each assay. Plastic Petri dishes (20 x 100 mm) were purchased from Baxter Diagnostics, Inc., Hayward, CA. hFGF-B was obtained from Clonetics Corporation, San Diego, CA.

20

For testing, a minimum amount of acetone-methanol (1:1) was added to the test compounds for sterilization. The acetone-methanol mixture was then evaporated to dryness in a sterile hood. The compounds were dissolved dimethyl sulfoxide (DMSO) first and then diluted with saline containing methylcellulose. The final concentrations were 2% DMSO and 0.5% methylcellulose. All test solutions were added to each CAM in 20-ml aliquots.

25

C. Development of the CAM for Measuring Angiogenesis Inhibition

The method of Folkman, et-al., *Dev. Biol.* 41: 391-394 (1974), with some modifications, was used to cultivate chicken embryos as follows:

30

Fresh fertile eggs were incubated for three days in a standard egg incubator. On Day 3, eggs were cracked under sterile conditions and embryos were placed in 20 x 100 mm plastic Petri dishes and cultivated at 37°C in an embryo incubator with a water reservoir on the bottom shelf. Air was continuously bubbled into the water reservoir by using a small pump so that the humidity in the incubator was kept constant.

35

Observations were made daily to ensure that all embryos were healthy. Dead or unhealthy embryos were removed from the incubator immediately to avoid contamination. On Day 9, a sterile silicone ring cup was placed on each CAM and 0.5

mCi of  $^{14}\text{C}$ -proline with or without the test compound plus 2.5 ng of hFGF dissolved in saline containing 0.5% methylcellulose was delivered into each ring cup in a sterile hood. 2-ME was tested in parallel to serve as a reference compound. After addition of test materials, the embryos were returned to the incubator and cultivation continued. On Day 5 12, all embryos were transferred to a cold room at 4-10°C. The antiangiogenic effect of each test compound was determined by using the collagenase assay (Maragoudakis, *et al.*, *J. Pharm. Exp. Ther.* 251:679-682 (1989)) to measure  $^{14}\text{C}$ -proline incorporation into collagenous protein.

10 D. Collagenase Assay for Measurement of  $^{14}\text{C}$ -Proline Incorporation into Collagenous Protein

With the embryos placed on ice, a piece of CAM 10 mm in diameter was cut off under each ring cup and placed in a separate tube. To each tube was added 1.0 ml of phosphate-buffered saline (PBS, pH 7.3) containing 0.11 mg cycloheximide and 0.17 mg dipyridyl. The tubes were placed in a boiling water bath for 10 min and then cooled to room temperature. The PBS in each tube was discarded after centrifugation at 3000 x g for 10 min. The CAM residue was washed once with 3 ml of 15% TCA and then three times with 3 ml of 5% TCA. Centrifugation was carried out as described above between each washing. At this point all non-protein bound radioactivity was removed, and the 15 CAM containing the newly synthesized  $^{14}\text{C}$ -collagenous protein was suspended in 0.9 ml of 0.1 N NaOH and 1.1 ml of HEPES buffer at pH 7.4. The pH of the sample was neutralized with 0.8 N HCl, using phenol red as indicator.

To digest the  $^{14}\text{C}$ -collagenous protein, 7.5 units of collagenase and 500 nmol of calcium chloride in 40 ml of HEPES buffer was added to the above samples, and 20 mixtures were incubated at 37°C for 4 h. The reaction was stopped by adding 1.0 ml of 20% TCA containing 5 mg of tannic acid into each tube. After vortex mixing, the samples were centrifuged at 3000 x g for 10 min. An aliquot of the clear supernatant was taken for scintillation counting to quantitate the radiolabeled tripeptides corresponding to basement membrane collagen and other collagenous materials synthesized by the CAM 25 from  $^{14}\text{C}$ -proline. The CAM pellets in each tube were solubilized in 0.5 ml of 1.0 N NaOH by boiling in a water bath for 5 min. An aliquot of the dissolved CAM was used for protein determination using the method provided by Pierce Chemical Co. (Instruction manual for protein assay using bicinchoninic acid (BCA) Pierce Chemical Co., Rockford, IL.). The radioactivity per milligram of protein from the CAM treated with a test 30 compound relative to that from the control CAM gave the percent of angiogenesis inhibition.

E. Results

Tables 1 and 2 summarize the results of the two separate experiments. BTO-956 showed statistically significant inhibitory effects on hFGF-B-induced angiogenesis. At 75 µg/CAM, the inhibition caused by BTO-956 was 30%, compared to 5 38% caused by the same concentration of the known antiangiogenic agent, 2-methoxyestradiol. The results from these two experiments suggest that the antiangiogenic effect of BTO-956 in the CAM assay is on the same order as that of 2-methoxyestradiol, a drug that is under development as an antiangiogenic agent.

Table 1

**INHIBITORY EFFECTS OF  
COMPOUNDS ON ANGIOGENESIS INDUCED BY hFGF-B**

Compound	Dose (µg/CAM)	<sup>14</sup> C-Proline Incorporated into Collagenous Protein (cpm/mg-protein) (mean ± S.E.)	% Inhibition
BTO-956	75	4374 ± 651	31
	25	5098 ± 785	19
	8.3	6884 ± 1395	0
2-Methoxyestradiol	75	3875 ± 891	38
	25	5068 ± 1609	20
	8.3	5711 ± 1469	9
Control	—	6300 ± 696	—

Table 2

**INHIBITORY EFFECTS OF  
COMPOUNDS ON ANGIOGENESIS INDUCED BY hFGF-B**

Compound	Dose ( $\mu\text{g/CAM}$ )	$^{14}\text{C}$ -Proline Incorporated into Collagenous Protein (cpm/mg-protein) (mean $\pm$ S.E.)	% Inhibition
BTO-956	75	7534 $\pm$ 1099 <sup>a</sup>	32
	25	9100 $\pm$ 1664	18
	8.3	10138 $\pm$ 1625	10
2-Methoxyestradiol	75	6984 $\pm$ 1022 <sup>b</sup>	38
	25	7303 $\pm$ 1424	35
	8.3	10499 $\pm$ 1372	6
Control	—	11200 $\pm$ 829	—

<sup>a</sup> Significantly lower than control, P < 0.02.

<sup>b</sup> Significantly lower than control, P < 0.01.

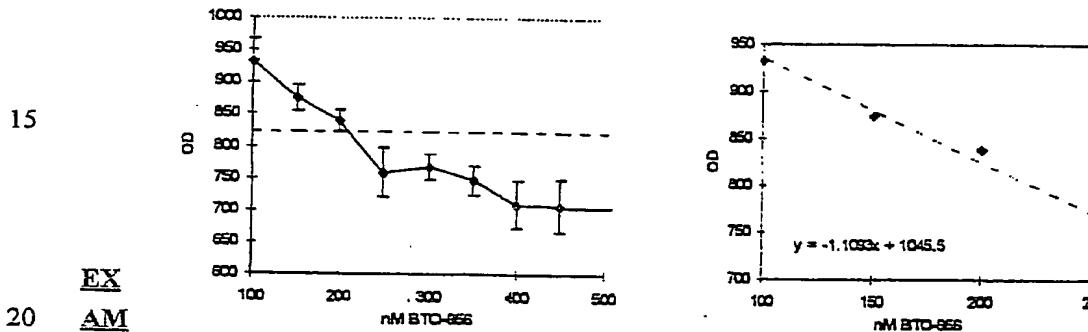
**EXAMPLE III**

This example illustrates the determination ID<sub>50</sub> of BTO-956 on the proliferation of human microvascular endothelial cells (HMVEC).

Human microvascular endothelial cells (HMVEC) (Clonetics Corporation, #CC-2505) were seeded into 96-well plates at a concentration of  $5 \times 10^3$  cells/well in a volume of 100  $\mu\text{l}$ /well of Endothelial Growth Medium (EGM-2-MV, Clonetics Corporation #CC-3162). Plates were incubated at 37°C in 5% CO<sub>2</sub> for 24 h and then covered with 100  $\mu\text{l}$ /well of EGM-2-MV. BTO-956 was diluted to 20 mM in dimethyl sulfoxide (DMSO) and further diluted with EGM-2-MV to 2x the concentrations reported below. Then 100  $\mu\text{l}$  aliquots of BTO-956/EGM-2-MV dilutions were added to the HMVEC preparations and plates were incubated at 37°C in 5% CO<sub>2</sub> for 3 days. The relative number of cells was determined by adding 20  $\mu\text{l}/\text{ml}$  of Alamar Blue (BioSource International #DAL-1025) for 3-6 h at 37°C and measuring color changes indicating metabolic activity by using a Millipore Cytofluor 2350 Fluorescence Measurement System at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. In this assay, the intensity of the fluorophore signal is directly proportional to cell number.

A broad range curve was first established for BTO-956 to determine the concentrations to use for a narrow range curve. A narrow range curve was then generated to find a region around the ID<sub>50</sub> point at which the curve is linear. The ID<sub>50</sub> was calculated by using the linear portion of the curve in the immediate area around the dose giving 50% inhibition and using the equation  $y = ax + b$ , where x is the calculated ID<sub>50</sub>, y is 50% of the maximum optical density (OD), and a and b are constants.

To establish an exact ID<sub>50</sub> for BTO-956, a narrow concentration range was tested on HMVECs (from 450 nM to 50 nM). Maximum inhibition was determined by using an additional upper concentration limit of 10  $\mu$ M. The baseline value was determined by using medium without compound. The ID<sub>50</sub> was determined to be 201 nM on Day 3.



#### PLEX IV

A. BTO-956 is orally bioavailable and has minimal normal tissue toxicity

Gavage administration of BTO-956 to nude mice at doses as high as 1,000 mg/kg-day for 60 to 180 days produced only slight and transient increases in liver and kidney weights. No other gross pathology was observed. No mortality was seen at a daily administration of 4,000 mg/kg-day of BTO-956 for 3 weeks. Because a common toxic effect of anti-proliferative chemotherapeutic agents is myelosuppression caused by depletion of bone marrow stem cells, we tested the possibility that BTO-956 may have bone marrow toxicity. Swiss Webster mice were exposed to the drug at 500 mg/kg-day by gavage for a period of 4 weeks, and bone marrow was examined histologically. The bone marrow profiles of treated animals did not differ from those of control animals exposed only to the carrier vehicle, and no hematopoietic abnormalities were found. Both myeloid and erythroid lineages were observed at various stages of maturation. These results demonstrate that oral administration of BTO-956 is well tolerated by animals exposed to the drug for periods of up to six months, and has no obvious myelosuppressive activity after one month of daily administration at a therapeutic dose. Preliminary studies

(not shown) indicate that therapeutic oral doses of BTO-956 are within the range of 500 to 1,000 mg/kg-day.

5           B.     BTO-956 has strong growth delay effects in human breast and ovarian carcinoma xenografts

Swiss NCr nude (nu/nu) female mice implanted with MDA MB-231 human breast carcinoma xenografts showed a strong growth delay response to oral treatment with BTO-956 (FIG. 1). Intraperitoneally administered cyclophosphamide (CTX; Cytoxan; 150 mg/kg), which is used in some regimens for adjuvant breast cancer 10 therapy was used as a positive control. Growth of tumors in the BTO-956-treated group was suppressed during 6 weeks of therapy while those in the control vehicle-treated animals increased approximately 5-fold on the average (relative mean tumor volume; FIG. 1A). BTO-956 was also effective in inhibiting the growth of OVCAR-3 human ovarian carcinoma xenografts (FIG. 1B). Using the same schedules and doses as those 15 described for the MDA MB-231 breast tumor study, but with shorter study duration, the relative mean tumor volume at 5 weeks of animals exposed to BTO-956 was again approximately 5-fold less than that of the vehicle-treated controls. Two important conclusions of this study are the following: (1) BTO-956 is effective as a cytostatic/cytotoxic antitumor drug when administered orally; and (2) BTO-956 is 20 essentially nontoxic even at very high orally administered doses.

C.     BTO-956 induces a mitotic arrest in MCF-7 human breast carcinoma cells

Having demonstrated significant antitumor effects of BTO-956 *in vivo*, we 25 investigated its cytotoxicity toward human breast carcinoma cells *in vitro*. The IC<sub>50</sub> (concentration for 50% reduction of cell number relative to that of a vehicle-treated control) of BTO-956 in MDA MB-231 and MCF-7 cell cultures treated for 48 h was 0.3 μM and 0.6 μM, respectively. Investigation of the effect of BTO-956 (1 μM for 48 h) on the nuclear morphology of these cells demonstrated that a significant number had 30 condensed or fragmented nuclei characteristic of apoptotic cell death. To test whether BTO-956 exerts its cytotoxicity by perturbing the cell cycle, MCF-7 cultures were exposed to the drug at 1 μM for 24 h (FIG. 2). A large fraction of these cells showed chromosome condensation characteristic of a prometaphase arrest (FIG. 2B). In agreement with this conclusion, treatment of MCF-7 cells with the antimitotic drug 35 colchicine (1 μM) for 24 h produced identical chromosome condensation patterns (FIG. 2C). After 48 h of exposure to 1 μM BTO-956, MCF-7 cells showed substantial cytotoxicity based on the criterion of loss of membrane integrity measured by a trypan blue exclusion assay. This result is consistent with that of the tumor growth delay study

described above, and demonstrates that BTO-956-induced cell death occurs subsequent to a prometaphase arrest in human breast carcinoma cell cultures.

To investigate the mitotic arrest caused by BTO-956 in more detail, flow cytometry was used to determine the effect of the drug on the cell-cycle progression of MCF-7 cells. These experiments showed that at least 60% of these cells arrested at G<sub>2</sub>/M within 24 h of treatment with 1 μM BTO-956. The same finding was obtained for MDA MB-231 cells exposed to the drug under the same conditions. These results demonstrate that BTO-956 causes human breast carcinoma cells to accumulate at the G<sub>2</sub>/M cell-cycle interphase, presumably by activating a mitotic spindle-assembly checkpoint. Such arrested cells eventually undergo cell death which can occur by an apoptotic mechanism.

D. BTO-956 inhibits microtubule dynamics both *in vitro* and in cultured cells

Microtubules are major cytoskeletal components composed of α- and β-tubulin heterodimers bound with GTP. Antimitotic drugs such colchicine, *Vinca* alkaloids, and paclitaxel (Taxol) perturb the intrinsic dynamic instability of microtubules arising from GTP hydrolysis by binding directly to tubulin and causing inhibition of spindle formation with consequent mitotic arrest. To determine whether the antimitotic effect of BTO-956 exposure could be explained by tubulin-binding activity, two studies were performed. The effect of the drug on microtubule assembly was investigated directly by using a cell-free fluorescence assay involving the visualization of microtubule formation from rhodamine-labeled tubulin and microtubule seeds in the presence of GTP. In this assay, 15 μM tubulin rapidly polymerized to form long, bright microtubule threads in the absence of a tubulin-binding agent (FIG. 3A), whereas in the presence of 1 μM colchicine microtubule formation was completely inhibited (FIG. 3B). The same effect was observed in the presence of 10 μM BTO-956 (FIG. 3D). At a concentration of 1 μM BTO-956 was less potent, but nonetheless produced much shorter structures than those visible in the control experiment (FIG. 3C). In a second study, the ability of BTO-956 to perturb cellular microtubule assembly was investigated by using HeLa cells (FIG. 4), which also arrest at mitosis when exposed to the drug (FIG. 4) shows that HeLa cells exposed to 10 μM BTO-956 for 1 h had aberrant microtubule networks resembling those created by exposure to 1 μM colchicine. Taken together, these findings demonstrate that BTO-956 can interact directly with tubulin *in vitro* and in cells to inhibit microtubule formation, much like the antimitotic drug colchicine.

E. BTO-956 competes with colchicine for binding to tubulin *in vitro*

Tubulin-binding antimitotic drugs interact with the protein at diverse sites. Colchicine binds to soluble tubulin heterodimers at a single high-affinity site (the

colchicine binding site) to form a kinetically inert complex. Vinblastine binds to one or two identical high-affinity sites on tubulin (*Vinca* alkaloid binding sites) that are different from the colchicine site. To determine whether the effect of BTO-956 on microtubule assembly is mediated by a specific site on tubulin, the ability of  $^{14}\text{C}$ -labeled drug to compete with colchicine or vinblastine for binding to purified tubulin was measured as a function of colchicine or vinblastine concentration. FIG. 5 demonstrates that colchicine inhibited the binding of BTO-956 to tubulin, whereas vinblastine had no effect. This finding indicates that BTO-956 interacts directly or indirectly with the colchicine site but not the *Vinca* alkaloid high-affinity binding sites of tubulin *in vitro*.

10

#### EXAMPLE V

This example demonstrates that BTO-956 can downregulate the expression of cytokines in lipopolysaccharide-stimulated murine macrophages. The following is an assay for measuring the ability of Compounds of Formula I to reduce the levels of Tumor Necrosis Factor (TNF- $\alpha$ ).

20           A.     Cell Line

The murine macrophage PU5-1.8 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in DMEM medium supplemented with 100 mM sodium pyruvate, 0.1mM nonessential amino acids, 2mM glutamine and 5% fetal bovine serum (Life Technologies, Staten Island, NY). Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub>-95% air at 37°C. Cells were passages twice weekly by firmly tapping the side of the flask to dislodge the adherent cells. Both nonadherent and adherent cells were passages. Exponentially growing cells were seeded at  $5 \times 10^5$ /mL, 4mL per 60-mm dish 24 h prior to the experiment. Test compounds were delivered in 1mL volumes of the medium added to each dish at the start of the experiment. All dishes were incubated at 37°C in 5% CO<sub>2</sub>-95% air for 3 h.

30           B.     Reagents

The Tumor Necrosis Factor (TNF- $\alpha$ ) cDNA was obtained from the ATCC (Rockville, MD). [ $\alpha$ -<sup>32</sup>P]-dCTP (250  $\mu\text{Ci}$ ) and nylon membranes (Hybond N) were obtained from Amersham (Arlington Heights, IL.). Colchicine (used as a control) was purchased from the Sigma Chemical Company (St. Louis, MO). Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from DIFCO Laboratories (Detroit, MI). All plastic supplies were from VWR Scientific products (San Francisco, CA).

C. Northern blotting

Total RNA was isolated by the guanidinium-cesium chloride method as described in N.S. Waleh, J. Gallo, T.D. Grant, B.J. Murphy, R.H. Kramer and R.M. Sutherland. (1994) "Selective downregulation of integrin receptors in spheroids of squamous cell carcinoma" *Cancer Res.*, 54:838-843. Five to 10 µg of total RNA was electrophoresed in 1% agarose gels containing 6% formaldehyde. Following electrophoresis, gels were stained with ethidium bromide to visualize the positions of 28S and 18S RNA. The RNAs were then transferred to nylon membranes (Amersham Hybond N) by capillary blotting and fixed to the filter by exposure to UV light. The blots were probed with <sup>32</sup>P-labeled cDNA sequences of human TNF- $\alpha$  obtained from the American Type Culture Collection (ATCC). The TNF- $\alpha$  cDNA was a 1.1 kb PstI fragment of plasmid pE4 in *E. coli* MM294 (ATCC 39894). Hybridizations were carried out at 42°C in 50% formamide, 5 X SSC, 5X Denhardt's solution, 0.1% SDS, and 0.3 mg/mL salmon sperm DNA. Filters were washed by 1 X SSC, 0.1% SDS, twice at room temperature for 15 min and once at 55°C in 0.1 X SSC, 0.1% SDS for 1 hr. Filters were exposed to X-ray film at -70°C using an intensifying screen (Coronex Hi-Plus).

Hybridized bands were quantified by analyzing the images obtained by using a video densitometer (Applied Imaging Corporation, Santa Clara, CA). Film densities were calibrated using an optical-density wedge.

20

D. RESULTS

Treatment of PU5-1.8 murine macrophages with LPS (100 ng/mL) for 3 h resulted in a significant increase (> 7 fold) in the level of TNF- $\alpha$  mRNA as determined by Northern blot analysis (see, Fig. 1). Treatment of cells with only BTO-956 or colchicine at 10 µM concentration had no effect on TNF- $\alpha$  mRNA expression. However, addition of colchicine or BTO-956 at 10 µM to the LPS treated cultures resulted in substantial reduction of TNF- $\alpha$  mRNA accumulation. The inhibition levels were 68% for colchicine and 51% for BTO-956, respectively.

To establish a concentration-effect relationship, macrophages were exposed to various concentrations of BTO-956 in the presence of the stimulus LPS for 3 h. Fig. 2 illustrates that the amounts of TNF- $\alpha$  mRNA declined with the increasing concentrations of BTO-956. The maximum effect was observed at 10 µM concentration of BTO-956. Total RNA was intact in cells treated with different concentrations of BTO-956 and colchicine, suggesting toxicity was not problematic.

The results indicate that BTO-956 at 10 µM partially suppresses, but does not completely inhibit, the expression of TNF- $\alpha$  mRNA in the murine macrophage cell

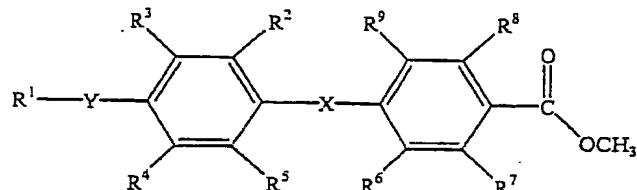
line PU5-1.8. This indicates that BTO-956 downregulates LPS-stimulated responses by affecting the microtubule-dependent signaling pathways.

This finding is of clinical interest because BTO-956 can be used as a new class of compounds to prevent LPS-mediated excessive TNF- $\alpha$  production and its 5 undesired side effects. This is especially attractive because BTO-956 has been shown to be a safe and well tolerated drug when administered orally to animals.

It is to be understood that the above description is intended to be 10 illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications and publications, are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1        1. A method of inhibiting angiogenesis in a tissue or organ, said  
 2        method comprising contacting said tissue or organ with an anti-angiogenic amount of a  
 3        compound having the formula:



4

5        or a pharmaceutically acceptable salt thereof;

6              wherein:

7        R<sup>1</sup> is C<sub>1</sub>-C<sub>6</sub>-alkyl;

8        R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 9        of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
 10      halogen, NO<sub>2</sub> and NH<sub>2</sub>;

11      R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected from the group  
 12      consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-  
 13      C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>; and

14              X, if present, is selected from the group consisting of oxygen, sulfur, -  
 15      CH<sub>2</sub>- and carboxy.

1        2. A method in accordance with claim 1 wherein:

2        R<sup>1</sup> is methyl or ethyl;

3        R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 4        of hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 5        halogen, NO<sub>2</sub> and NH<sub>2</sub>;

6        R<sup>6</sup>, R<sup>7</sup>, are each independently selected from the group consisting of  
 7        hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 8        halogen, NO<sub>2</sub> and NH<sub>2</sub>;

9        R<sup>8</sup> and R<sup>9</sup> are iodine; and

10              X, if present, is selected from the group consisting of oxygen, sulfur, -  
 11      CH<sub>2</sub>- and carboxy.

1        3. A method in accordance with claim 1 wherein the compound is  
 2        methyl 3,5-diido-4-(4-methoxyphenoxy)benzoate.

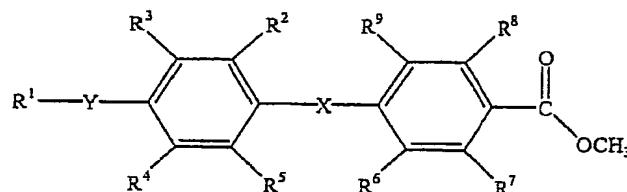
1           4. A method in accordance with claim 1 wherein said tissue or organ  
 2 is in a mammalian subject.

1           5. A method in accordance with claim 1 wherein said compound is  
 2 formulated in a pharmaceutically acceptable form with an excipient or carrier.

1           6. A method in accordance with claim 1 wherein said compound is  
 2 formulated in a liposome.

1           7. A method in accordance with claim 6 wherein said liposome is  
 2 conjugated to a targeting moiety which is specific for endothelial cells.

1           8. A method of treating a mammalian disease associated with  
 2 undesired and uncontrolled angiogenesis, said method comprising administering to a  
 3 mammal an anti-angiogenic amount of a compound having the formula



4  
 5 or a pharmaceutically acceptable salt thereof;  
 6        wherein:

7        R<sup>1</sup> is C<sub>1</sub>-C<sub>6</sub>-alkyl;

8        R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 9 of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
 10 halogen, NO<sub>2</sub> and NH<sub>2</sub>;

11       R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected from the group  
 12 consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-  
 13 alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>; and

14       X, if present, is selected from the group consisting of oxygen, sulfur, -  
 15 CH<sub>2</sub>- and carboxy.

1           9. A method in accordance with claim 8 wherein:

2        R<sup>1</sup> is methyl or ethyl;

3        R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 4 of hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 5 halogen, NO<sub>2</sub> and NH<sub>2</sub>;

35

6           R<sup>6</sup>, R<sup>7</sup>, are each independently selected from the group consisting of  
 7   hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 8   halogen, NO<sub>2</sub> and NH<sub>2</sub>;

9           R<sup>8</sup> and R<sup>9</sup> are iodine; and

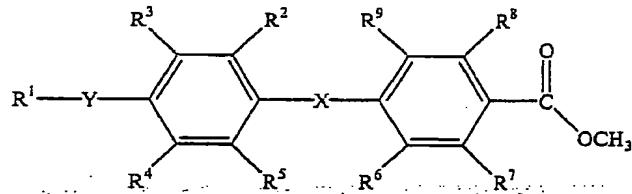
10          X, if present, is selected from the group consisting of oxygen, sulfur, -  
 11   CH<sub>2</sub>- and carboxy.

1           10. A method in accordance with claim 8 wherein the compound is  
 2   methyl 3,5-diido-4-(4-methoxyphenoxy)benzoate.

1           11. A method in accordance with claim 8 wherein said mammalian  
 2   disease is a member selected from the group consisting of arthritis, atherosclerotic  
 3   plaques, diabetic retinopathy, neovascular glaucoma, trachoma and corneal graft  
 4   neovascularization, psoriasis, scleroderma, hemangioma, hypertrophic scarring, vascular  
 5   adhesions and angiofibroma.

1           12. A method in accordance with claim 8 further comprising the step  
 2   of determining the inhibition of said undesired and uncontrolled angiogenesis by tissue  
 3   biopsy.

1           13. A method of inhibiting the vascularization of endothelial cells, said  
 2   method comprising contacting a tissue or organ comprising said endothelial cells with an  
 3   anti-angiogenic amount of a compound having the formula



5   or a pharmaceutically acceptable salt thereof;

6           wherein:

7           R¹ is C<sub>1</sub>-C<sub>6</sub>-alkyl;

8           R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 9   of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
 10   halogen, NO<sub>2</sub> and NH<sub>2</sub>;

11          R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected from the group  
 12   consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-  
 13   alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>; and

14                    X, if present, is selected from the group consisting of oxygen, sulfur, -  
 15                  CH<sub>2</sub>- and carboxy.

1                    14. A method according to claim 8 wherein:

2                    R<sup>1</sup> is methyl or ethyl;

3                    R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
 4                  of hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 5                  halogen, NO<sub>2</sub> and NH<sub>2</sub>;

6                    R<sup>6</sup>, R<sup>7</sup>, are each independently selected from the group consisting of  
 7                  hydrogen, C<sub>1</sub>-C<sub>4</sub>-alkyl, C<sub>2</sub>-C<sub>4</sub>-alkenyl, C<sub>2</sub>-C<sub>4</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>4</sub>-alkoxy,  
 8                  halogen, NO<sub>2</sub> and NH<sub>2</sub>;

9                    R<sup>8</sup> and R<sup>9</sup> are iodine; and

10                  X, if present, is selected from the group consisting of oxygen, sulfur, -  
 11                 CH<sub>2</sub>- and carboxy.

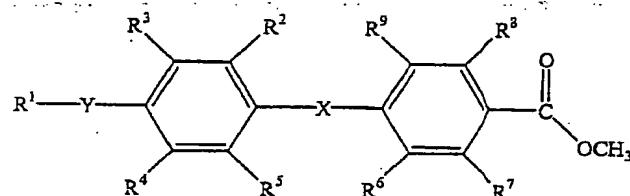
1                    15. A method according to claim 13 wherein the compound is methyl  
 2                 3,5-diido-4-(4-methoxyphenoxy)benzoate.

1                    16. A method according to claim 13 wherein the endothelial  
 2                 vascularization is in noncancerous tissue or organs.

1                    17. A method according to claim 13 wherein said compound is  
 2                 formulated in a liposome and said liposome is conjugated to a targeting moiety which is  
 3                 specific for endothelial cells.

1                    18. A method of inhibiting the growth of a tumor in a mammal, said  
 2                 method comprising:

3                    (a) administering to said mammal an anti-angiogenic amount of a  
 4                 compound having the formula:



5                    or a pharmaceutically acceptable salt thereof;

6                    wherein:

7                    R<sup>1</sup> is C<sub>1</sub>-C<sub>6</sub>-alkyl;

37

R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
halogen, NO<sub>2</sub> and NH<sub>2</sub>:

**R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup>** are each independently selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>;

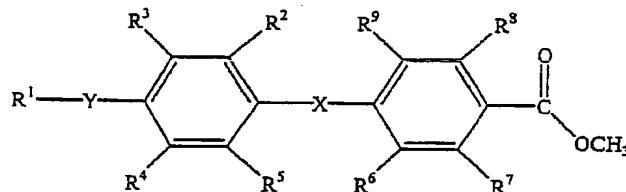
15 X, if present, is selected from the group consisting of oxygen, sulfur, -  
16 CH<sub>2</sub>- and carboxy; and

(b) histologically observing the vascularization of said tumor, thereby determining the inhibition of tumor growth

1                   19. A method of inhibiting the growth of a tumor in a mammal in  
2 accordance with claim 18 wherein said administration to said mammal is carried out with  
3 immunotherapy.

1                   20. A method of inhibiting the growth of a tumor in a mammal in  
2 accordance with claim 19 further comprising the step of administering to said mammal a  
3 tumor vaccine.

1                   21. A method for reducing the level of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )  
2 produced by a cell, said method comprising contacting said cell with a compound having  
3 the formula:



5 or a pharmaceutically acceptable salt thereof;

wherein:

7                   R<sup>1</sup> is C<sub>1</sub>-C<sub>6</sub>-alkyl;  
8                   R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
9 of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
10 halogen, NO<sub>2</sub> and NH<sub>2</sub>;

R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected from the group consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>; and

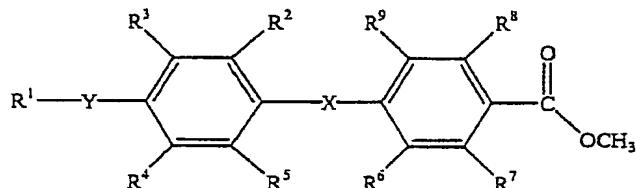
14                            X, if present, is selected from the group consisting of oxygen, sulfur, -  
15    CH<sub>2</sub>- and carboxy.

1                   22. A method according to claim 21 wherein the compound is methyl  
2 3,5-diiodo-4-(4-methoxyphenoxy)benzoate.

1                   23. A method according to claim 21 wherein said compound is  
2 formulated in a liposome.

1                   24. A method according to claim 21 wherein said compound is  
2 formulated in a liposome and said liposome is conjugated to a targeting moiety which is  
3 specific for tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) or a receptor to said tumor necrosis factor  $\alpha$ .

1                   25. A method for treating an inflammatory disease, said method  
2 comprising a therapeutically effective amount of a compound having the formula



4 or a pharmaceutically acceptable salt thereof:

5 wherein:

6 R<sup>1</sup> is C<sub>1</sub>-C<sub>6</sub>-alkyl:

<sup>7</sup> R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are each independently selected from group consisting  
<sup>8</sup> of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl, C<sub>1</sub>-C<sub>6</sub>-alkoxy,  
<sup>9</sup> halogen, NO<sub>2</sub> and NH<sub>2</sub>:

10 R<sup>6</sup>, R<sup>7</sup>, R<sup>8</sup> and R<sup>9</sup> are each independently selected from the group  
11 consisting of hydrogen, C<sub>1</sub>-C<sub>6</sub>-alkyl, C<sub>2</sub>-C<sub>6</sub>-alkenyl, C<sub>2</sub>-C<sub>6</sub>-alkynyl, aryl, hydroxyl,  
12 C<sub>6</sub>-alkoxy, halogen, NO<sub>2</sub> and NH<sub>2</sub>; and

13 X, if present, is selected from the group consisting of oxygen, sulfur, -  
14 CH<sub>2</sub>- and carboxy.

1/6

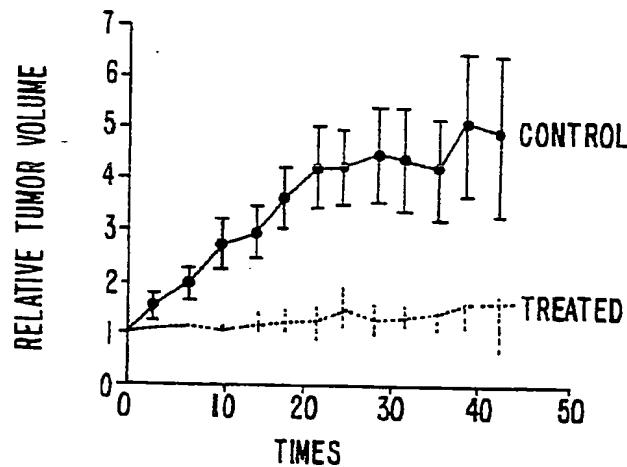


FIG. 1A.

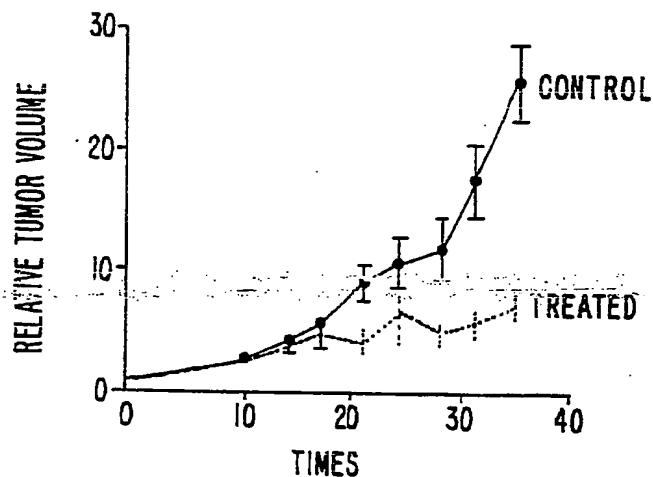


FIG. 1B.

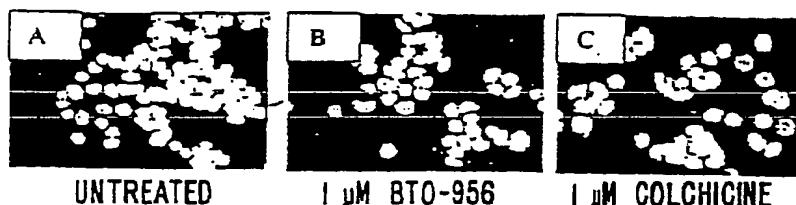


FIG. 2A.

FIG. 2B.

FIG. 2C.

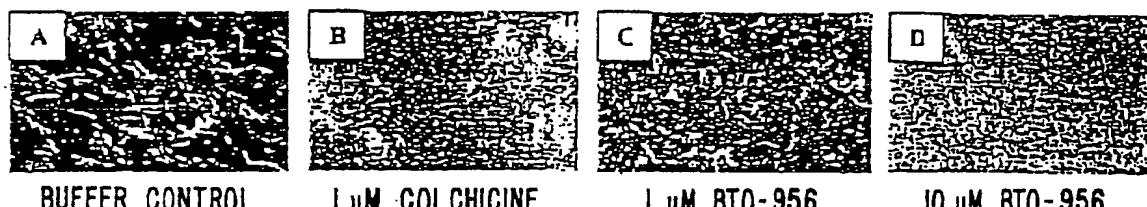
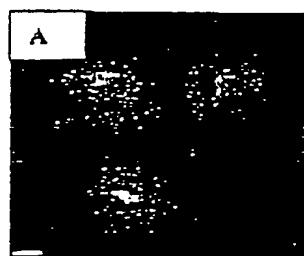


FIG. 3A.

FIG. 3B.

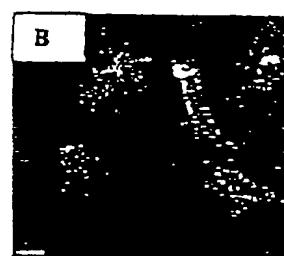
FIG. 3C.

FIG. 3D.



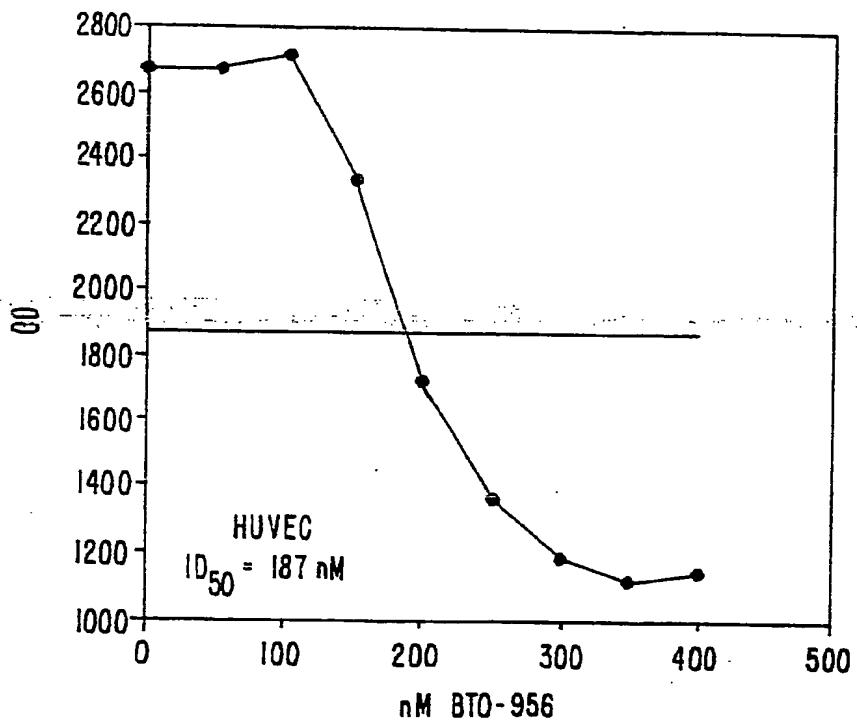
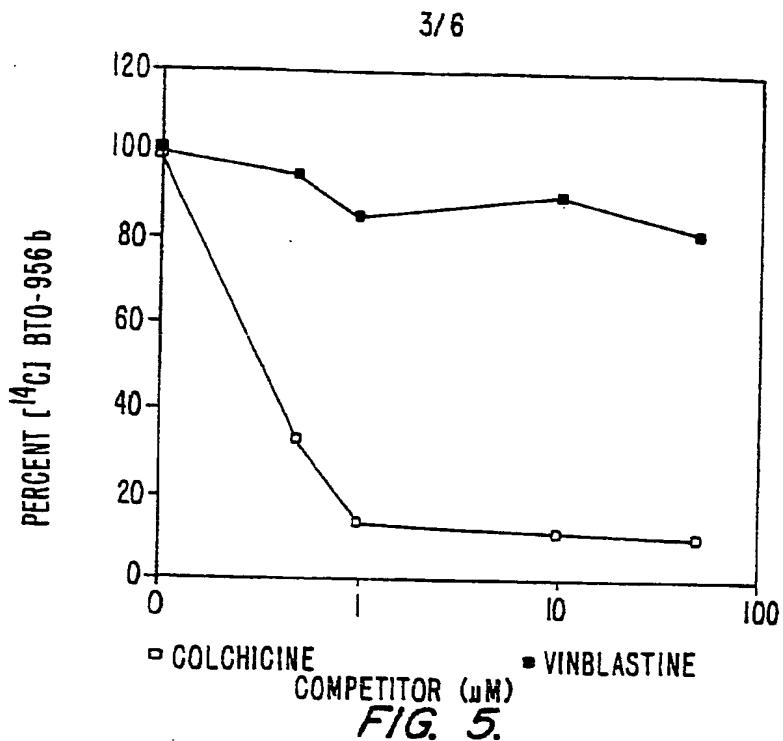
UNTREATED CELLS

FIG. 4A.

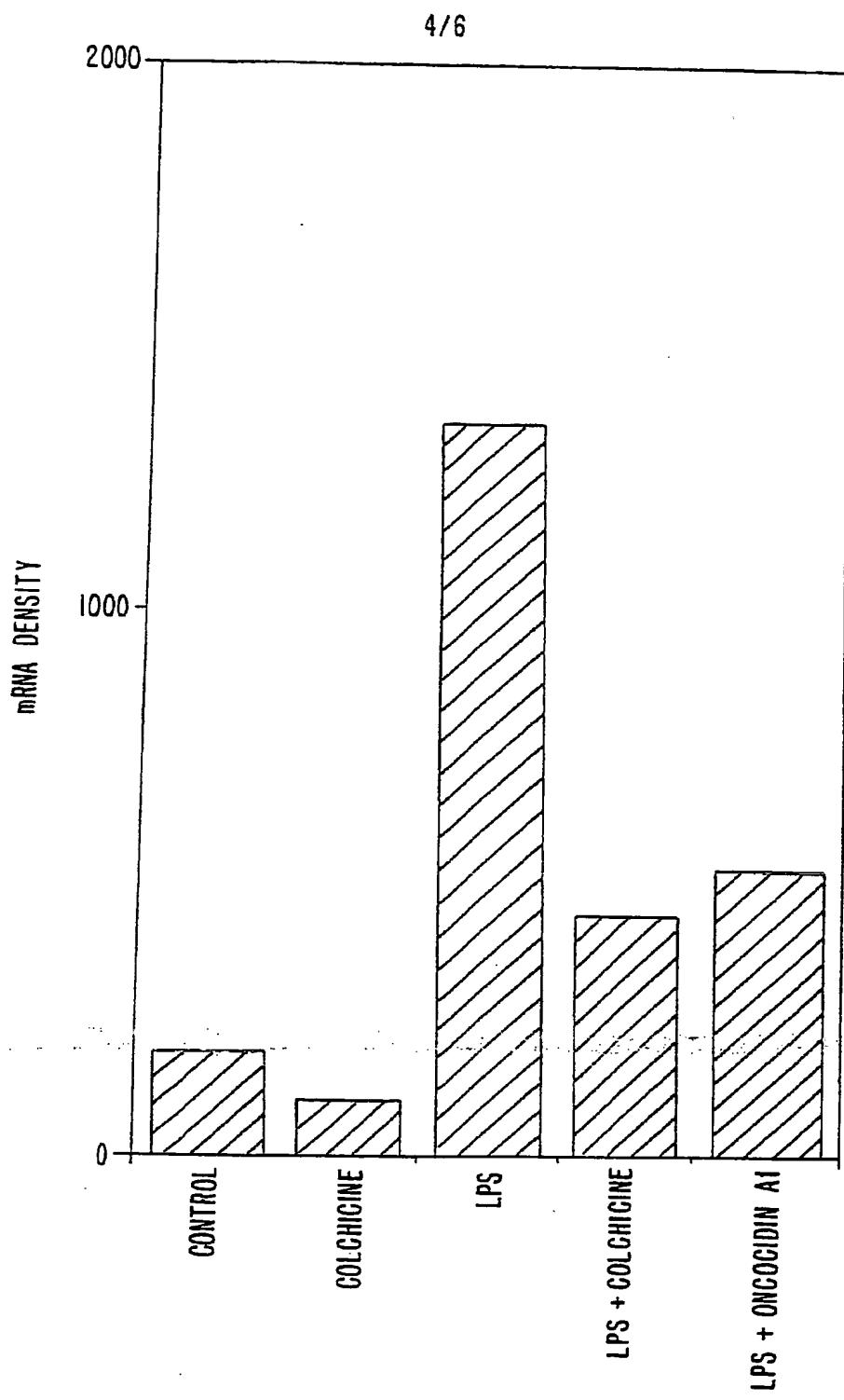


BTO- 956

FIG. 4B.



SUBSTITUTE SHEET (RULE 26)



*FIG. 7.*  
SUBSTITUTE SHEET (RULE 26)

5/6

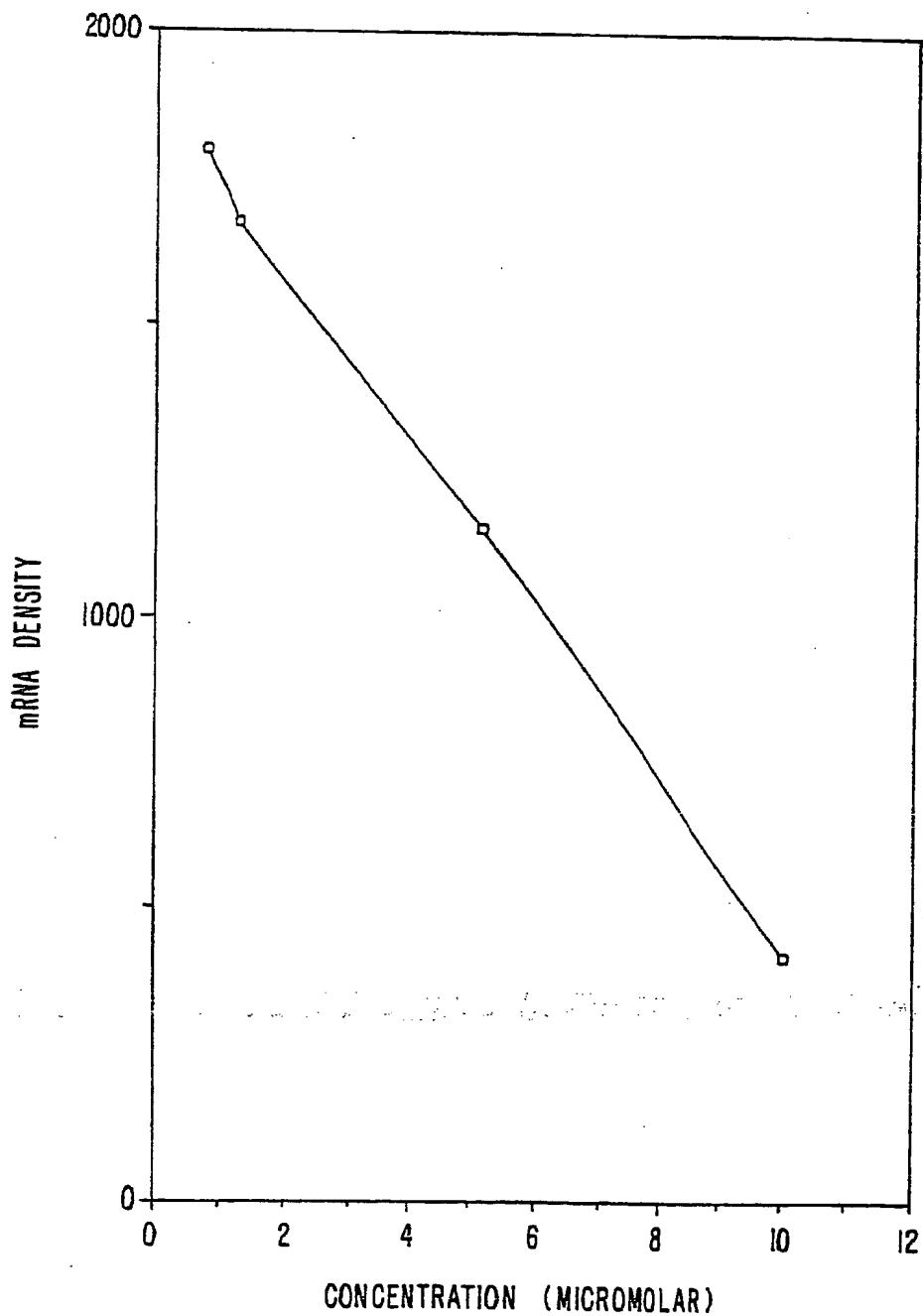
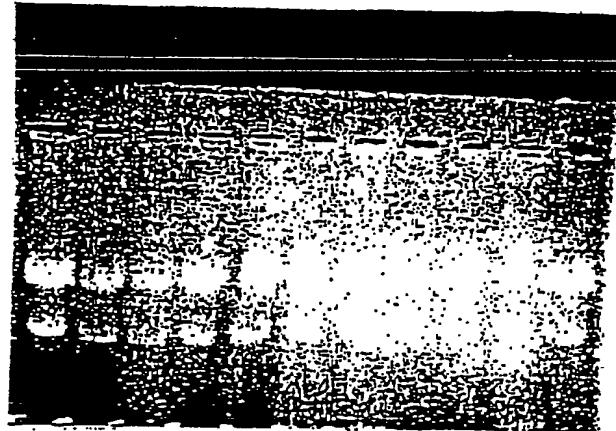


FIG. 8.

6/6



*FIG. 9.*

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/06220

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K31/235

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MENDELEYEV ET AL: "structural specificity and tumoricidal action of DIME" INT. J. ONCOL., vol. 10, no. 4, 1997, pages 689-695, XP002113317 page 690; table I page 692; table II ---	8-10, 13-15, 18
X	WO 97 46228 A (OCTAMER INC) 11 December 1997 (1997-12-11) claims 1-9 ---	8-10, 13-15, 18 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*&\* document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 August 1999	03/09/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patenlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer  Trifilieff-Riolo, S

## INTERNATIONAL SEARCH REPORT

In national Application No  
PCT/US 99/06220

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	KUN ET AL: "molecular pharmacology of DIME and its non-hydrolyzable ethanone analog (DIPE)" INT. J. MOL. MED., vol. 2, no. 5, 11 November 1998 (1998-11-11), pages 585-590, XP002113318 page 590, paragraph 5 -----	8-10, 13-15,18
X,P	CHEN ET AL: "oncocidin A1: a novel tubulin-binding drug with antitumor activity against human breast and ovarian carcinoma xenografts in nude mice" BIOCHEM. PHARMACOL., vol. 56, no. 5, 1 September 1998 (1998-09-01), pages 623-633, XP002113319 abstract -----	8-10, 13-15,18
A	MASUDA ET AL: "thyroxine related compounds" TAKEDA KENKYUSHO HO, vol. 30, no. 3, 1971, pages 466-474, XP002113320 -----	8-11
A	TRIPP ET AL: "synthesis of methylene- and carbonyl-bridged analogs of iodothyronines and iodothyroacetic acids" J. MED. CHEM., vol. 16, no. 1, 1973, pages 60-64, XP002113321 page 62, right-hand column, line 16 - line 22 -----	1-25
E	WO 99 20263 A (OCTAMER INC) 29 April 1999 (1999-04-29) claims 1-9 -----	8-10, 13-15,18

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 99/06220

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 1-25 because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: - because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically.  
See FURTHER INFORMATION SHEET PCT/ISA/210
3.  Claims Nos.: - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1, 8, 13, 18, 21 and 25 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds cited in claims 2, 3, 9, 10, 14, 15 and 22.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 99/06220

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9746228	A 11-12-1997	US 5736576 A		07-04-1998
		AU 3218297 A		05-01-1998
WO 9920263	A 29-04-1999	US 5922775 A		13-07-1999
		AU 1195499 A		10-05-1999